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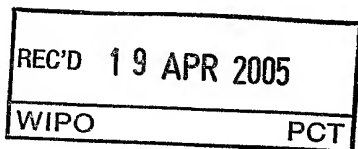
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PCT REQUEST

Original (for SUBMISSION)

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| 0-5 | Petition The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty | |
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| 0-7 | Applicant's or agent's file reference | BLOcp263-113 |
| I | Title of Invention | GENETICALLY MODIFIED PLANTS AND THEIR APPLICATIONS IN PHYTOREMEDIATION. |
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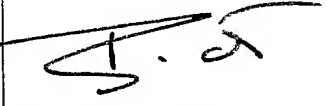
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| IV-1 | Agent or common representative; or address for correspondence The person identified below is hereby/ has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: | agent | |
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| V | DESIGNATIONS | | |
| V-1 | The filing of this request constitutes under Rule 4.9(a), the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents. | | |
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| VIII-2 | Declaration as to the applicant's entitlement, as at the international filing date, to apply for and be granted a patent | - | |
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| IX | Check list | number of sheets | electronic file(s) attached |
|--------------|--|---|------------------------------------|
| IX-1 | Request (including declaration sheets) | 4 | - |
| IX-2 | Description (excluding sequence listing part) | 33 | - |
| IX-3 | Claims | 3 | - |
| IX-4 | Abstract | 1 | ✓ |
| IX-5 | Drawings | 13 | - |
| IX-7a | Sub-total number of sheets | 54 4 55 4 | |
| IX-6a | Sequence listing part of description filed in computer readable form in addition to being filed in paper form (under Section 801(a)(ii)) | 7 | - |
| IX-7 | TOTAL | 61 | |
| | Accompanying Items | paper document(s) attached | electronic file(s) attached |
| IX-8 | Fee calculation sheet | ✓ | - |
| IX-16(A) | Sequence listing in computer readable form: | | |
| IX-16(A)-(i) | copy submitted for the purposes of international search under Rule 13ter only (and not as part of the international application) | - | 1 Diskette |
| IX-17 | PCT-SAFE physical media | - | ✓ |
| IX-18 | other: | Declaration | |
| IX-19 | Figure of the drawings which should accompany the abstract | | |
| IX-20 | Language of filing of the international application | English | |
| X-1 | Signature of applicant, agent or common representative |  | |
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GENETICALLY MODIFIED PLANTS AND THEIR APPLICATIONS IN PHYTOREMEDIATION

The instant invention relates generally to the field of phytoremediation. More particularly the invention relates to genetically modified plants able to
5 accumulate heavy metals in shoots and to methods of removing and possibly recovering said heavy metals, using said genetically modified plants.

Numerous ecological and health problems are associated with environmental contamination. Heavy metals contamination of soils is a particular hazard, due to the almost indefinite environmental persistence of metals. While some heavy
10 metals are naturally present in soil, heavy metal contamination is often the result of human activities. Contamination of the environment with heavy metals has increased drastically due to industrialization: mining, smelting, intensive agriculture, sludge dumping, energy and fuel production, electroplating and fuel exhaust contribute to contaminating the soil with heavy metals.

15 The soils contaminated with heavy metals inhibit normal plant growth and cause contamination of foodstuffs. Many heavy metals are very toxic to human health and carcinogenic at low concentrations. Therefore removal of heavy metals from the environment is crucial.

Currently, the most common methods for dealing with this problem
20 remain removal and burial of the contaminated soil or the isolation of the contaminated area. However, such methods are extremely expensive.

One relatively new option for decontaminating soil is phytoremediation, which uses plants to scavenge organic and inorganic contaminants from the soil.

25 Phytoremediation may be divided into phytoextraction, rhizofiltration and phytostabilization:

- Phytoextraction is a method using contaminant-accumulating plants to extract said contaminants from soil into the harvestable parts of the plants;
- Rhizofiltration is a method using plant roots to remove contaminants from polluted aqueous streams; and
- 30 - Phytostabilization is the stabilization of contaminants such as toxic metals in soils to prevent their entry into ground water, also with plants.

However, usually most plants are unable to grow in soil containing high levels of heavy metals.

Certain plants have been found to accumulate nickel, cobalt, copper, lead, zinc, cadmium and selenium. Accumulators of nickel are by far the most common amongst the hyper accumulators discovered to date. The more rarely absorbed heavy metals include manganese, cadmium and lead, with lead being particularly hard to absorb as well as being very difficult to translocate from plant roots into plant stems after it is absorbed.

Therefore, there is a need for plants being able to grow in soils containing high concentrations of heavy metals. To confer heavy metal resistance to plants, it has been proposed to use the properties of heavy metal-transporting P-type ATPase and more specifically the P_{1B}-ATPases which are known to be heavy metal transporters.

The P_{1B}-ATPases are transporters that use the energy liberated in the exergonic ATP hydrolysis reaction to translocate positively charged substrates across membranes (Inesi, 1985). P_{1B}-ATPases are found in all living organisms, from archaea to humans and are thought to transport soft metal cations. They are referred to as HMAs (Axelsen and Palmgren, 2001; Williams et al., 2000) or CPx-type ATPases (Solioz and Vulpe, 1996; Rensing et al., 1997; Williams et al., 2000).

In the yeast *Saccharomyces cerevisiae*, only 2 P_{1B}-ATPases have been found (Catty et al., 1997), CCC2 and PCA1, both participating in copper transport (Fu et al., 1995; Bassett Jr et al., 1996; Ran et al., 1994).

In *Arabidopsis thaliana*, seven P_{1B}-ATPases were previously identified (Axelsen and Palmgren, 2001) but the recent genome release revealed an eighth gene belonging to the HMAs group (P-type ATPase database; <http://biobase.dk/~axe/Patbase.html>).

The recent completion of two draft sequences of the rice (*Oryza sativa*) genome shows a similar number of enzymes within this group. Compared with other eukaryotes, which only possess one or two P_{1B}-ATPases, the parallel expansion of the number of HMAs in *Arabidopsis* and *Oryza* suggests that these enzymes play important roles in the overall movements of metals in plants.

In both species, these eight proteins have been classified in 6 clusters, based on full-length sequence alignments and intron positions (Baxter et al., 2003). In *Arabidopsis*, the two first clusters (HMA1, HMA2-4, respectively) are possible Zn/Cd/Co/Pb ATPases; the four others have a potential role in Cu/Ag transport. The first two characterised enzymes are members of this last subgroup. AtHMA7 (RAN1) has been identified as a copper transporter to ethylene receptors (Hirayama et al., 1999; Woeste and Kieber, 2000). Recently AtHMA6 (PAA1) has been characterised as a Cu transporter, participating in the translocation of this metal to chloroplastic copper proteins (Shikanai et al., 2003). Also, all eukaryotic HMAs characterised so far belong to this Cu/Ag subgroup.

Despite many members of the Zn/Cd/Co/Pb subgroup being known (Rensing et al., 1998; Hou and Mitra, 2003; Lebrun et al., 1994a and 1994b; Tsai et al., 2002), the first characterization of one homologue in plants has only just been published (Mills et al., 2003). These Authors showed that AtHMA4 induced an increased tolerance to Cd in wild-type *Saccharomyces cerevisiae* and restored Zn resistance of the *Escherichia coli zntA* mutant, which is highly sensitive to this metal. As predicted, AtHMA4 was unable to restore copper tolerance in the *copA* mutant context.

Therefore, it has been proposed to use the properties of these transporters in view to essentially increase resistance of plants to heavy metals:

- European Patent Application 1 136 558 proposes plants including sequences encoding one or more heterologous heavy metal transport and/or sequestration proteins of various prokaryotic or eukaryotic origins. The metal transport proteins are, for instance, membrane proteins such as P-type ATPases and preferably a bacterial P-type ATPase, such as cadmium ATPase. It is specified that such plants have improved heavy metal tolerance and possibly heavy metal accumulation; however, as regards the use of cadmium ATPase modified plant, the increased resistance of the plant to cadmium is the only characteristic pointed out.

- The PCT International Application WO 02/081707 discloses plant transformants with enhanced heavy metal resistance and decreased uptake of heavy metals. The P-type ATPase used to transform the plants is the ZntA, that is a P-type ATPase of *E. coli*, which pumps Pb(II)/Cd(II)/Zn(II) across the plasma membrane.

More specifically it is considered, in said PCT International Application that biologically active ZntA-like heavy metal pumping ATPases include zinc-transporting ATPase (NC_000913), zinc-transporting ATPase (NC_002655), heavy metal-transporting ATPase (NC_003198), P-type ATPase family (NC_003197), cation
 5 transporting P-type ATPase from *Mycobacterium leprae* (GenBank #Z46257) and many others may be used. ZntA-transgenic plants and for instance ZntA-transgenic *Arabidopsis* plants are described; said plants showed enhanced resistance to lead and cadmium and the content of lead and cadmium was lower than in a wild-type plant. Therefore such transformed plants can grow in an environment contaminated with
 10 heavy metals; thus, said technique can be useful for generating crop plants with decreased uptake of harmful heavy metals. This is confirmed by the article in the name of LEE J. et al. (Plant Physiol., 2003), Inventors of said PCT International Application, which confirm that a bacterial heavy metal pump, ZntA, can be expressed in *Arabidopsis*, resulting in increased resistance to Pb(II) and Cd(II) and
 15 reduced heavy metal contents in the shoots of the transgenic plants. Therefore, both documents show that it is feasible to develop plants, i.e. transgenic ZntA *Arabidopsis* plants, with enhanced resistance and reduced uptake of heavy metals. They conclude that if ZntA can be introduced into crop plants, the resulting plants would be safer because they would contain less heavy metal in their shoots.

20 Therefore, the aim of the transgenic plants described in these two documents is not phytoremediation; thus the transgenic plants proposed in said documents cannot be used for phytoremediation and more specifically for phytoextraction, as defined here above.

- Mills R.F et al, (Plant J., 2003, precited) showed that AtHMA4, a
 25 P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass induced an increased tolerance to Cd in wild-type *Saccharomyces cerevisiae* and restored Zn resistance of the *Escherichia coli* zntA mutant, which is highly sensitive to this metal. Even though they consider that there is considerable interest in metal transport in plants because of the implications for phytoremediation, it is only shown, in said document, that
 30 AtHMA4 is mainly expressed in roots of *Arabidopsis* and that it could function in planta as a detoxification system in epidermal root cells, existing at the plasma

membrane to pump excess metals such as Zn or Cd into the rhizosphere. Therefore, they propose to use plants containing AtHMA4 for rhizofiltration.

It emerges from the foregoing that none of the disclosed methods proposes an efficient phytoremediation system for removal of heavy metals from soil, i.e. a method using the phytoextraction process, which is particularly adapted to heavy metal removal from soil. Indeed, the prior disclosed methods have focused on the selection of plants that have a lower uptake of heavy metals than the wild-type, and that maintain healthy growth even in an environment contaminated with heavy metals and not on phytoremediation by phytoextraction.

Moreover, despite ever increasing interest and research in the field, several problems associated with phytoremediation remain. For instance, many metal-accumulating plants are unable to translocate a substantial proportion of the metal they acquire from their roots to other tissues.

Therefore, there is a need for plants able to extract heavy metals in harvestable parts thereof, in view to propose a cost-effective phytoremediation of heavy metals by phytoextraction.

The Inventors have found that, unexpectedly, plants overexpressing at least one P_{1B} -ATPase of the subfamily or subclass of the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ transporters are good candidates for their use in a method of phytoremediation using the phytoextraction process, knowing that such genetically modified plants translocate efficiently the heavy metals in the shoots.

Therefore, in one aspect of the invention, genetically modified plants are provided which are able to accumulate heavy metals and translocate them to the shoots and more specifically to the stems and the leaves.

The preferred embodiments of this aspect of the invention include genetically modified plants, characterized in that they include more than one copy of a sequence encoding a P_{1B} -type ATPase of the $Zn^{2+}/Co^{2+}/Cd^{2+}/Pb^{2+}$ subclass and that they overexpress said P_{1B} -type ATPase.

According to the invention, said P_{1B} -type ATPase is selected from the group consisting of heavy metal ATPase HMA1 to HMA4 of *Arabidopsis thaliana* (i.e. AtHMA1, AtHMA2, AtHMA3 and AtHMA4), whatever the ecotype. The

corresponding sequences are available on the following websites: <http://mips.gsf.de> or <http://biobase.dk/~axe/Patbase.html>.

The nucleic sequences which may advantageously be used are the ones of *Arabidopsis thaliana*:

5 . ecotype Ws (Wassilewskija): AY054390 (AtHMA2), AY434729 or AY055217 (AtHMA3), AF412407 (AtHMA4));

. ecotype Columbia: NM_119890 (AtHMA1), NM_119157 or AY434728 (AtHMA2), NM_119158 or AY434730 (AtHMA3), NM_127468 or AJ297264 (AtHMA4);

10 . ecotype Landsberg: AJ400906 (AtHMA1).

However, the invention includes also the use of corresponding sequences of other wild ecotypes of *Arabidopsis thaliana*. The Man skilled in the Art may easily retrieve said equivalent sequences to the ones specified hereabove (AtHMA1-4) by appropriate alignment with said sequences.

15 According to another embodiment of the invention, said genetically modified plant includes more than one copy of at least two different sequences encoding two different P_{1B}-type ATPases of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass.

According to yet another embodiment of the invention, said genetically modified plant includes more than one copy of a sequence encoding a P_{1B}-type
20 ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass and at least another sequence selected among sequences encoding (1) an enzyme involved in metal chelation (phytochelatase synthase, glutathion synthetase or gamma-glutamylcystein synthase) or (2) another metal transporter such as YCF1 or other ABC transporters.

Preferably the at least two copies of said AtHMA sequence are
25 inserted in an appropriate vector, said AtHMA sequences being operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence, such as a plant specific promoter and more preferably the CaMV35S promoter and said vector is introduced in the selected plants with known methods. Said vector is advantageously an *Agrobacterium*/plant shuttle vector.

30 Preferably said vector may be introduced in plants, such vector allowing the transformation of plants with the *Agrobacterium tumefaciens* technology.

According to the invention, the plants to be used are selected in the group consisting of *Brassica juncea*, *Poplar*, *Nicotiana tabacum*.

The instant invention also relates to a recombinant vector able to transform plants, characterized in that said vector includes more than one copy of at least a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass (heavy metal pumping ATPases) in view that said transformed plants overexpress at least one of said P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass.

Advantageously, said coding sequences are operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence, such as a plant specific promoter.

The invention also relates to plant cells or plants transformed by said vector.

The instant invention also relates to a method of producing genetically modified plants which overexpress at least a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass, comprising:

- preparing a recombinant vector as defined here above, i.e. comprising more than one copy of at least a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass, operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence and
- introducing said recombinant vector into a plant cell or plant tissue to produce a genetically modified plant cell or a genetically modified plant tissue.

In another aspect of the invention, methods are described involving the genetically modified plants as described above in the extraction of heavy metals from soils. Therefore, the instant invention also relates to a method of phytoremediation of heavy metals from soil, characterized in that it includes:

- a step of planting genetically modified plants as described here above, in an area containing soil contaminated with at least one heavy metal and
- collecting and removing plant tissues from said genetically modified plants at appropriate time intervals.

The preferred embodiments of this aspect of the invention involve the extraction of one of the following heavy metals: Zn, Co, Cd or Pb, from soil.

The entire plant might be removed after it is allowed to grow on metal-containing soil incorporating those metals into its tissues. However, in many of the preferred embodiments, it may be taken advantage of the ability of said genetically modified plants as described here above to sequester substantial proportions of its accumulated metal in stems and leaf tissues.

Thus, said genetically modified plants are planted in contaminated soil and allowed to grow and scavenge the cited metals from the soil.

Then, at appropriate time intervals, the metal containing tissues and more preferably leaves and possibly branches are removed from the plant, allowing the remaining plant tissues to survive.

The collected plant tissues are removed from the growing area and properly disposed, so that the metal containing tissues are not allowed to reassimilate in the soil.

In another embodiment of the instant invention, said heavy metals may be extracted in the M^{n+} state from said plant tissues by known methods.

In yet another embodiment of the instant invention, said heavy metals are extracted from ashes obtained after having burnt the collected metal containing tissues, said metal being in the M^0 state.

The burning operation is made under controlled environmental process.

The heavy metals, obtained in a M^0 state, may be, advantageously, directly recycled.

The Table below establishes the correspondences between the sequence numbers as they appear in the sequence listing and the name of the various sequences.

| Designation | SEQ ID |
|----------------------|--------------|
| Rev6HMA4 | SEQ ID NO:1 |
| Rev5HMA4 | SEQ ID NO:2 |
| Rev4HMA4 | SEQ ID NO:3 |
| Rev3HMA4 | SEQ ID NO:4 |
| Rev2HMA4 | SEQ ID NO:5 |
| Rev1HMA4 | SEQ ID NO:6 |
| 1HMA4 | SEQ ID NO:7 |
| Rev14HMA4 | SEQ ID NO:8 |
| TopoFV1for | SEQ ID NO:9 |
| TopoFV1rev | SEQ ID NO:22 |
| HMA4For230 | SEQ ID NO:10 |
| Rev2bisHMA4 | SEQ ID NO:11 |
| Actin8F | SEQ ID NO:12 |
| Actin8R | SEQ ID NO:13 |
| Rev18HMA4 | SEQ ID NO:14 |
| 10HMA4 | SEQ ID NO:15 |
| Rev8HMA4 | SEQ ID NO:16 |
| 22HMA4 | SEQ ID NO:17 |
| Δ HisStopHMA4 | SEQ ID NO:18 |
| HMA4D401Afor | SEQ ID NO:19 |
| HMA4D401Arev | SEQ ID NO:20 |
| 9HMA4 | SEQ ID NO:21 |

In addition to the preceding arrangements, the invention also comprises other arrangements which will emerge from the description which follows, which refers to exemplary embodiments of the method which is the subject matter of the present invention as well as to the appended drawings, in which:

- **Figure 1:** Nucleic and amino acid sequences of AtHMA4 from the *Ws* ecotype (accession number AF412407). The grey highlighted characters correspond to the 5' UTR. The N-terminal heavy metal motif, the numerous cysteine doublets and the His stretch are boxed; the highly conserved sites are underlined. The amino acid sequence deleted in the alternative spliced form is black highlighted. The position of the stop insertion for the *Athma4 Δ His* form and its GFP fusion is indicated by the solid black vertical arrow while the empty one corresponds to the GFP fusion position of *AtHMA4* and *Athma4as*.

- **Figure 2:** Evidence for the existence of an alternative spliced form of the cDNA of *AtHMA4* in the *Ws* ecotype, absent in *Ler* and *Col-0* ecotypes. RT were carried out with 4 μ g total leaf RNA.

- **Figure 3:** A: Western blot analysis of the microsomal fractions of the different transformant strains expressing the chimeric constructions and AtHMA4 without fused EGFP. 50 µg protein was loaded on 8 % SDS-PAGE for each fraction. The resolved proteins were electrotransferred on nitrocellulose membrane and revealed with anti-EGFP horseradish peroxidase antibodies. B: Confocal microscopy observations of yeast cells expressing AtHMA4::EGFP. Top, transmission image; bottom, EGFP fluorescence image.

- **Figure 4:** A: AtHMA4, but not Athma4D401A, Athma4as and Athma4ΔHis, confers an increased tolerance to Cd and is able to functionally substitute the *ycf1* mutant strain of *S. cerevisiae*. The various strains were grown on solid SC minimal medium, supplemented with CdCl₂ at the indicated concentrations. Plates were incubated at 30 °C for 4 days. Upper part (wild-type): pYES2 only-, AtHMA4-, Athma4D401A-, Athma4as- and Athma4ΔHis-transformants of the wild-type strain BY4741. Bottom part: same as upper part, except that the constructs are expressed in the *ycf1* mutant strain (*ycf1* YCF1 indicated a complementation of the mutant strain *ycf1* by pYES2-YCF1). B: Metal tolerance conferred by AtHMA4 does not implicate a glutathione S-conjugated form of Cd. The different strains were grown on solid SC minimal medium without (control) or supplemented with 70 µM CdCl₂ and 5 mM BSO during 4 days at 30 °C.

- **Figure 5:** Expression of AtMHA4 or AtHMA4::EGFP, but not Athma4ΔHis, induces a functional substitution of *ycf1* in the presence of Pb. The strains were grown on solid SC minimal medium without (control) or supplemented with 20 mM Pb(CH₃COO)₂ (conditions and strains were the same as in Fig. 4).

- **Figure 6:** AtHMA4 confers a slightly enhanced tolerance to Zn and was able to partially substitute the *zrc1* strain. Strains and culture conditions were the same as in Fig. 4. ZnSO₄·7H₂O was added to the medium at the indicated concentrations.

- **Figure 7:** Cd content of a 48 h culture of pYES2-only (WT) or AtHMA4- (WT *AtHMA4*) transformant of the wild-type; YCF1- (*ycf1* YCF1) or AtHMA4- (*ycf1* *AtHMA4*) transformant of the mutant *ycf1* strain. Error bars represent the SE from five independent experiments.

- **Figure 8:** Expression profile of *AtHMA4* (and *Athma4as*) determined by RT-PCR on RNA extracted from various tissues. The results of RT-PCR are presented after 27 and 30 cycles to better visualise the expression of the alternative spliced form (*Athma4as*, fragment of 350bp size).

5 - **Figure 9:** **A:** Exon/intron map of *AtHMA4* including the 5'UTR and position of the T-DNA insertion of the Rm396 mutant. The asterisk on the ninth exon (including the 5'UTR) indicates the position of the deletion in the alternative spliced form of the cDNA (*Athma4as*). The arrows indicate the different primers used to control the existence of a transcript by RT-PCR, using RNA extracts from Rm396
10 and Ws. **B:** The T-DNA insertional Rm396 line is a true K.-O. mutant for *AtHMA4*. The RT-PCR was carried out on RNA with different primer pairs, specific to *AtHMA4*. Upper: oligonucleotides 1 (1HMA4: SEQ ID NO:7) and 2 (Rev6HMA4: SEQ ID NO:1); middle: 1 and 3 (Rev18HMA4: SEQ ID NO:14); lower: 4 (10HMA4: SEQ ID NO:15) and 5 (Rev8HMA4: SEQ ID NO:16).

15 - **Figure 10:** Expression profile of GUS activity *in plants*. a: roots; b: root slice; c: plantlet; d: stem and cauline leaf; e: stamens.

 - **Figure 11:** *AtHMA4* expression is up-regulated by Zn treatment. **A:** RT-PCR amplification on total RNA. **B:** GUS staining on root system. The inset corresponds to a binocular observation of a 1mM Zn-treated root. Plants were grown
20 hydroponically and treated for 24 h with ZnSO₄ at the indicated concentrations.

 - **Figure 12:** The overexpression of *AtHMA4* confers an increased tolerance to metals. **A:** Overexpression of *AtHMA4* determined by RT-PCR carried out with RNA extracted from leaves. **B:** Zn content of leaves from wild-type (Col-0) or overexpressing (35S-*AtHMA4*) plants hydroponically grown during 8 days on
25 standard nutrient solution (Zn 3 µM), determined by ICP measurements. **C:** Root length of vertically grown plantlets. The photography of plates are realised 15 days after germination. **D:** The graph represents the results of one representative experiment of 3 with one of the two lines analyzed in detail. Values are mean of measurements of 75-100 roots, and error bars represent SEM (P < 0.005).

30 It should be clearly understood, however, that these examples are given solely by way of illustration of the subject of the invention and do not in any manner constitute a limitation thereto.

EXAMPLE 1: MATERIALS AND METHODS**- Plant material, culture media, and extraction and measurement of total RNA.**

Plants were grown in a controlled-environment (8 h photoperiod of
 5 300 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$, 22 °C and 70 % relative humidity), in Murashige and Skoog
 medium which consisted of a ½ strength (MS/2) nutrient solution (Murashige and
 Skoog, 1962), plus 1 % (w/v) sucrose, and 0.7 % (w/v) Bacto agar to produce a solid
 medium. Germination of surface-sterilized seeds of *Arabidopsis thaliana*, ecotypes
 Wassiliewskija (Ws), Columbia (Col-0) and Landsberg *erecta* (Ler) occurred on the
 10 solid medium. After 2 weeks, the young plants were placed on sand, saturated with the
 same nutrient solution, for an additional 3 weeks period and finally transferred to a
 hydroponic culture system. When plants were treated with different metals (*in vitro* or
 hydroponically), they were supplied at various concentrations as indicated in the
 figure legends, in addition to that in the nutrient solution.

15 Total RNA was extracted from various tissues (root, leaf, stem,
 cauline leaf, flower and silique) according to Verwoerd et al. (1989), and the RNA
 concentration determined using UV absorption at 260 nm (BioPhotometer, Eppendorf,
 Hamburg, Germany).

- Cloning of the cDNA of AtHMA4

20 Two successive 5' RACE-PCR were performed (5' RACE System,
 GibcoBRL®, Carlsbad, CA) according to the manufacturer's instructions. In the first
 cycle of RACE, the cDNA was amplified with the gene specific oligonucleotide
 primer Rev6HMA4 (5'-GACCAATATGTTGATGTCGATCC-3') (SEQ ID NO:1)
 situated in the 2nd predicted exon. The two successive semi-nested PCR were carried
 25 out using the Expand High-Fidelity System (Roche, Mannheim, Germany) with the
 primers Rev5HMA4 (GGCTTTGGCAAGAATCGGATAG-3') (SEQ ID NO:2) and
 Rev4HMA4 (5'-GCGGCAACTGCTGCCACGGCGAGCC-3') (SEQ ID NO:3), respec-
 tively. Reaction conditions were 3 min at 94 °C, and then 35 cycles for 30 sec at 94
 °C, 30 sec at 53 °C or 67°C, 1 min at 68 °C. The last cycle was followed by an
 30 extended elongation step of 5 min at 68 °C. The amplification fragments were cloned
 in pGEM-T® Easy vector (Promega, Madison, WI). The second round of 5' RACE
 was performed as described above. The different primers used were Rev3HMA4 (5'-

CTTCTTCACTTTCTTTTTCTCTTCTTC-3) (SEQ ID NO:4) for the cDNA amplification, Rev2HMA4 (5'-GTAGCAAAAGGAAGAAGCCGATG-3') (SEQ ID NO:5) and Rev1HMA4 (5'-CTGGTTTGGTGGCGATCAGATAAAGG-3') (SEQ ID NO:6) were used in the two successive semi-nested PCR, with annealing temperature of 55 °C and 58°C, respectively.

First-strand full-size cDNA synthesis was performed with the ThermoScript™ RT-PCR system (Invitrogen™, Carlsbad, CA) according to the manufacturer's instructions using 4 µg of total leaf RNA and an oligo (dT)₂₀ primer. PCR was then carried out with Platinum® Pfx (Invitrogen™) with primers corresponding to the 5'-UTR and 3' end of the predicted coding sequence present in the BAC clone (5'-end primer 1HMA4 (5'-CACTTCTCTCAACCTTTATCTGAT-3') (SEQ ID NO:7) and 3'-end primer Rev14HMA4 (5'-GTTATTCAATCAATCTCCATCAAG-3') (SEQ ID NO:8)). The PCR reaction conditions were as described above, except the hybridization temperature (50 °C) and the final elongation step (10 min). The amplification fragment of 3.6 kb was directly cloned in the pCR®-XL-TOPO vector (Invitrogen™). The same procedure was used for the cloning of the cDNA of the alternative spliced form (*AtHMA4as*).

- Obtaining of *AtHMA4* overexpressing lines

After digestion by *EcoRV*, the CaMV35S cassette was subcloned in the *EcoRV* / *StuI* sites of the pGreen0179 binary vector. A *SmaI* site was added at the 3'-end of *AtHMA4* by cloning the reverse-complement primer pairs TopoFV1for (SEQ ID NO:9)/rev (SEQ ID NO:22) in the *NorI* site of the pCR®-XL-TOPO vector. The cDNA of *AtHMA4* was then extracted by *BamHI* / *SmaI* digestion and cloned at the same sites in the pGreen-CaMV35S vector. This construction was introduced by electroporation in AGL1 cells of *A. tumefaciens*. The agrotransformation of plants was carried out by the floral dip method (Clough and Bent, 1998). The transformant plants were selected on solid medium supplemented with hygromycin B 30 µg / ml.

- DNA fragment resolution

The DNA fragments were electrophoresed on 1 % (w/v) agarose gels prepared in TAE buffer in the presence of ethidium bromide (Sambrook et al., 1989). The DNA was visualised under UV light using a GeneGenius GG-X apparatus

(Syngene, Cambridge, UK). Extraction and purification of DNA fragments were obtained with the NucleoSpin® Extract kit (Macherey-Nagel, Düren, Germany).

- Sequencing

The "BigDyes" method (PE Biosystems, Foster City, CA) was used
5 with a Perkin Elmer ABI Prism 310 sequencer.

- Expression analysis of AtHMA4

* *RT-PCR*. Total RNA extractions and first-strand cDNA synthesis were carried out as described above. The same amount of RNA (4 µg) from all the organs analysed was used for RT-PCR with an oligo(dT)₂₀ primer. Primers
10 HMA4For230 (5'-CGCAGCTTGCTTTACTGGGTATCAAGTGTTGAAAG-3') (SEQ ID NO:10) and Rev2bisHMA4 (5'-CATCTAAAACATTCCCTAGCTGTTCTTGAGC-3') (SEQ ID NO:11) were used for PCR performed in the same conditions as described above, except the hybridization temperature (65 °C) and the number of cycles (26 cycles), which was chosen to be in the mid-log phase of amplification yield. As a
15 control, PCR of *actin8* gene was carried out on the same cDNA samples using the primers Actin8F (5'-GTGGTCGTACAACCGGTATTGTGTTGGACTCTGGTG-3') (SEQ ID NO:12) and Actin8R (5'-ACGCTGTAACCGGAAAGTTTCTCACATAGTGCACAAATGAC-3') (SEQ ID NO:13). These primers were added to the PCR reaction tubes 5 cycles later. The experiment
20 was repeated 3 times on independently isolated material. Three RT-PCR were carried out, to control the Rm396 mutant line, with primer pairs located upstream, on both sides and downstream relative to the T-DNA insertion position. The RT was carried out with the gene specific primer Rev6HMA4 (SEQ ID NO:1) for the first one; the first strand cDNA was used in PCR reaction with the primers 1HMA4 (SEQ ID NO:7) and Rev6HMA4 (SEQ ID NO:1). The PCR reactions for the other two were
25 performed with oligo(dT)₂₀ cDNA using primer pairs 1HMA4 (SEQ ID NO:7)/ Rev18HMA4 (5'-GCCTTTTGTGGAGCTAAGCTC-3') (SEQ ID NO:14) and 10HMA4/ Rev8HMA4 (5'-ATGGAGGCAGCAGCAGTTGTGTTCC-3' (SEQ ID NO:15)/ 5'-GGGACAACCACTGACTAACACAAC-3' (SEQ ID NO:16)), respectively. PCR of the
30 *actin8* gene was carried out on the same cDNA samples as described above.

* *GUS activity*. Plants or organs at different stage of their development were examined for GUS activity according to the procedure described by

Jefferson et al., 1987. The samples were immersed in the GUS staining solution (50 mM NaPO₄, pH 7.0, 0.01 % (w/v) Triton-X100, 1 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆ and 1 mg.ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc)), vacuum-infiltrated and incubated at 37 °C for 6 hours. Pigments were then washed out by incubation of stained sample in 2-3 changes of 70 % (v/v) ethanol at 60 °C for 2 h. Plantlet roots were desiccated by successive ethanol baths of rising concentrations, for 30 min each and then fixed in resin, before transverse sections were cut.

- Yeast strains, growth conditions and transformation

The *S. cerevisiae* reference strain BY4741 (*MATα*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*), and mutant strains *Δycf1* (like BY4741 except *YDR135c::kanMX4*), *Δzcr1* (like BY4741 except *YMR243c::kanMX4*) and *Δcot1* (strain BY4742: *MATα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*; *YOR316c::kanMX4*) were provided from Euroscarf (Frankfurt, Germany). All yeast strains were grown in YPD medium (1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose). Synthetic complete (SC) minimal medium lacking uracil was used for the selection and maintenance of yeast strains transformed with plasmid (0.67 % (w/v) yeast nitrogen base, 0.19 % (w/v) drop-out without uracil, 2 % (w/v) dextrose) (all products from Sigma, St Louis, MO).

AtHMA4 and the alternative spliced form (*Athma4as*) were cloned into the *NotI* site of the yeast expression vector pYES2 under a galactose-inducible promoter. A truncated version of *AtHMA4* lacking the C-terminal His₁₁ stretch was constructed. A PCR, using the *PfuUltra*TM (Stratagene®, Cedar Creek, TX), was carried out with the primers:

- 22HMA4: (5'-GAGAGTGTTGGAGACTGCAAGTCTGGTCATTGCCAGAAG-3')
(SEQ ID NO:17) and

- ΔHisStopHMA4: (SEQ ID NO:18)

(5'-AAGGAAAAAAGCGGCCGCAAAAGGAAAATTAGCTGCATAACTCTTTTGCATAAC-3'). This 860 bp fragment, digested by *BsgI* and *NotI*, was cloned in place of the same fragment in the full-size cDNA. This cDNA was then digested by *EagI* and cloned in pYES2 into the *NotI* site. These 3 cDNA were also cloned in the same vector in frame with the *EGFP* gene in 3'. For these clonings, the *AtHMA4* and *Athma4as* cDNA were first digested by *DraIII* at the 3' end of the genes, the end

being blunted by S1 nuclease treatment. Then the fragments were excised from the pCR[®]-XL-TOPO vector by *Eco*RI digestion and finally cloned into the *Eco*RI and *Sma*I sites of the pYES2-GFP vector, in frame with the *EGFP* gene. Also, to create the *Athma4ΔHis::EGFP* fusion, a PCR amplification was carried out on the cDNA of *AtHMA4* with the primers 22HMA4 (SEQ ID NO:17) and ΔHisHMA4 (5'-AAGGAAAAAAGCGGCCGCAAAAGGAAAACCCGGGGCTGCATAACTCTTTTGCAT AAC-3') (SEQ ID NO:18) which delete the His coding fragment and create a *Sma*I site. The amplification fragment was cloned in place of the corresponding fragment in the native cDNA into the *Bsg*I and *Not*I sites. The cDNA of *Athma4ΔHis* was then cloned into the *Eco*RI and *Sma*I sites of the pYES2-GFP vector. By sequencing, the fusions were controlled for the 3 constructions. The *Δycf1* mutant strain was complemented by a *YCF1::EGFP* fusion cloned into the *Sac*I and *Not*I sites of the pYES2 vector. A site-directed mutagenesis was carried out using the QuickChange[®] II XL Site-directed Mutagenesis Kit (Stratagene[®]) on the *AtHMA4::EGFP* construct in pYES2 as a template with the primers pairs:

- HMA4D401Afor: (SEQ ID NO:19)
(5'-GATCAAGATTGTTGCTTTCGCTAAACTGGGACTATTACAAGAGG-3')

and

-HMA4D401Arev: (SEQ ID NO:20)
(5'-CCTCTTGTAATAGTCCCAGTTTTAGCGAAAGCAACAATCTTGATC-3')

to create a mutant where the aspartate-401 residues from the conserved domain DKTGT was replaced by an alanine, giving the mutant *Athma4D401A::GFP*.

The yeast strains were transformed with pYES2 alone or with *AtHMA4*-, *Athma4D401A*-, *Athma4as*-, *Athma4ΔHis*-, *AtHMA4::GFP*-, *Athma4D401A::GFP*-, *Athma4as::GFP*- or *Athma4ΔHis::GFP*-pYES2, according to the manufacturer instructions (Invitrogen[™]).

- Assays for metal tolerance of yeast

Yeast cells were first pre-cultured overnight at 30 °C in SC minimal medium without uracil (2 % (w/v) raffinose instead of dextrose), then transferred to the induction medium at an O.D._{600nm} = 0.4 for 24 h (in same minimal medium except that 1 % (w/v) raffinose and 2 % (w/v) galactose were added). For solid media, 2 % (w/v) agar was added. The different metal solutions were added to the liquid or solid

media at the concentrations indicated in the figure legends. For the drop-tests on solid media, 2 μ L of the yeast cultures at $O.D._{600nm} = 2.0$ were spread ($1.6 \cdot 10^4$ cells) and three 10-fold serial dilutions were made. Plates were incubated at 30 °C for 4 days. For the liquid cultures, yeast cells were diluted to $O.D._{600nm} = 0.4$ and grown at late
5 log phase.

- GFP fluorescence observations by confocal microscopy

The different transformant yeast strains were observed for GFP fluorescence using a confocal laser scanning microscope (CLSM, Fluoview Olympus, France) fitted with a krypton/argon laser. Fluorescence excitation was achieved with a
10 dichroic filter (488 nm) and recording performed at 510 – 550 nm. Observations were carried out with an objective lens (UplanApo) at a magnification of x100 / 1.35 na, under oil immersion.

- Measurement of metal content of yeast cells

Yeast cells were grown in the same conditions as described for metal
15 tolerance assays. After 48 h of growth in 15 mL of induction medium supplemented with heavy metals, the OD_{600nm} was determined. The cells were collected by centrifugation, then washed three times with 10 mM EDTA, pH 7.5, and finally with water. The washed pellet was dried overnight at 80 °C and then mixed with nitric acid for mineralization using a MARS X microwave apparatus (CEM, Matthews, NC). Finally,
20 heavy metals contents were determined using an ICP-AES Vista MPX apparatus (Varian, San Diego, CA).

- Preparation of soluble and microsomal protein fractions

An overnight pre-culture of transformed yeast was carried out in 3 mL of SC-URA minimal medium at 30 °C, 270 rpm. Cells were harvested by
25 centrifugation (5 min, 6,000 rpm), diluted at 0.4 OD_{600nm} in 50 mL induction medium, and were grown for further 24 h. All further procedures were carried out at 4 °C. Yeast cells were harvested by centrifugation (10 min at 7,000 g), and suspended in 20 mL TEK buffer (50 mM Tris HCl pH 7.5, 1mM EDTA, 100 mM KCl). After centrifugation for 10 min at 7,000 g, the pellet was suspended in 2 mL TES buffer (50
30 mM Tris HCl pH 7.5, 1 mM EDTA, 0.6 M sorbitol, 1% (w/v) BSA, 2mM β -mercaptoethanol, 10 mM Na_2SO_4 , and a "Complete" protease inhibitor cocktail (Roche Diagnostics)). Cells were then broken by vigorous mixing with acid-washed

glass beads (425-600 μm , Sigma). The glass beads were washed four times with TES buffer, the washing fractions pooled and then centrifuged for 15 min at 8,000 rpm. Then, the supernatant was ultracentrifuged for 45 min at 100,000 g and the microsomal fraction was suspended in 500 μl TEG buffer (50 mM Tris HCl pH 7.5, 1mM EDTA, 30% (v/v) glycerol). Protein concentrations were measured with the
5 Bradford method (Sigma).

- SDS-PAGE and immunoblotting

Protein samples (50 μg) were rapidly thawed and mixed with denaturing buffer (1/4; v/v) containing 20 mM Tris HCl pH 8.0, 2% SDS (w/v), 10%
10 β -mercaptoethanol (v/v), 20% glycerol (v/v), and bromophenol blue. Samples were heated at 60 $^{\circ}\text{C}$ for 30 min, cooled, and then loaded on a 8% SDS-PAGE. After electrotransfer of the proteins onto a nitrocellulose membrane (BioRad, Hercules, CA), the blot was incubated with a primary monoclonal antibody raised against the EGFP diluted to 1/1000 (v/v) (Clontech, Palo Alto, CA). Revelation was obtained
15 with a secondary horseradish peroxidase antibody diluted to 1/5000 (v/v) (Sigma) and the SuperSignal[®] West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). All steps were performed according to the manufacturer's instructions.

EXAMPLE 2: RESULTS

- Cloning and sequence analysis of *AtHMA4* from the Ws
20 ecotype.

A study was conducted on the Wassiliewskija ecotype (Ws) since a T-DNA insertional mutant of *AtHMA4* was identified in the INRA Versailles collection. It was first determined whether the predicted ATG in the BAC clone AC002392 corresponded to the one in Ws. Two successive 5' RACE-PCR on total RNA
25 extracted from plant tissues were performed (see Example 1). In the first one, the cDNA was obtained with a reverse primer designed in the second predicted exon. By two successive semi-nested PCR, two amplification products were resolved, with 516 bp and 488 bp sizes, respectively. By sequencing, it was verified that these fragments correspond to *AtHMA4*. The second round of 5'RACE, using primers corresponding
30 to the new found sequence, did not enable another upstream transcription fragment to be obtained indicating that the 5' end of the mRNA of *AtHMA4* was determined. In addition to the coding sequence predicted from the genomic DNA of Columbia (Col-

0) ecotype, a 99 bp 5'-UTR was found. The full-length cDNA (3618 bp) was then obtained by a two steps RT-PCR in a unique fragment, cloned in the pCR®-XL-TOPO vector and sequenced (Genbank accession number AF412407) (Fig. 1). Some nucleic acid differences with respect to the sequence of the BAC clone from Col-0 were observed. The ecotypic differences between Ws and Col-0 were controlled by various independent PCR on genomic DNA and cDNA. Finally, four base changes were detected, resulting in amino acid differences (H vs. Q in position 537; K vs. R in 805; N vs. T in 970 and K vs. T in 1056).

Several RT-PCR experiments pointed to an alternative spliced form of the cDNA of *AtHMA4* (Fig. 2). With the same pair of primers, two amplification fragments were obtained with a size of about 450 bp and 350 bp, respectively. These two fragments were cloned and sequenced (442 and 358 bp), both corresponding to *AtHMA4*. To exclude any problem during the reverse transcription reaction, such as a stable hairpin loop of the mRNA, various conditions were tested (different reverse transcriptases and/or reverse primers more or less closely located downstream of the alternative splicing part of the gene). In all conditions tested, the spliced product was obtained in which a 84 bp fragment, between positions 1652 and 1735 of the nucleic sequence, was absent (Fig. 1, bases in square brackets and highlighted amino acids). The canonical limits of intron (5'-begin GT, 3'-end AG) were present at both sides of this fragment but different software for exon/intron prediction (GeneFinder, GeneScan) did not indicate this sequence as a potential intron. In the spliced form of the transcript the reading frame was conserved. Using RT-PCR, the full-size cDNA corresponding to this spliced form was amplified, cloned in the pCR®-XL-TOPO vector, and called *Athma4as*. Such spliced form of the cDNA was never observed with RNA extracted from the Landsberg *erecta* or Columbia ecotypes (Fig. 2), although the nucleic acid sequences around the splicing sites were identical for Col-0 and Ws.

AtHMA4 expression in yeast

AtHMA4, *Athma4D401A* and both truncated forms, *Athma4as* and *Athma4ΔHis*, were subcloned in the pYES2 vector and expressed in the *Saccharomyces cerevisiae* wild-type yeast strain, BY4741.

AtHMA4::GFP localization

To localise AtHMA4, a C-ter fusion of the different forms were also cloned in the pYES2 vector and expressed in the wild-type yeast strain BY4741. By Western blot using anti-EGFP horseradish peroxidase antibodies, the expression of the different chimeric proteins were controlled (Fig. 3A). The fusion proteins were observed in the microsomal fraction. Only one hybridization band which is in the range of the theoretical molecular masses of the fusion proteins (150-155 kDa) was found. No signal was observed with the microsomal fraction when the empty vector pYES2 was used, nor with AtHMA4 without EGFP, or in the soluble fraction. Under confocal microscopy, AtHMA4::EGFP appeared as a punctuated staining, typical of vesicles containing membrane proteins from the late Golgi apparatus, a pattern similar to the one described for CCC2 from *S. cerevisiae* and *Candida albicans*, respectively (Yuan et al., 1997; Weissman et al., 2002) (Fig. 3B). A similar pattern was observed with the 3 other forms of Athma4::EGFP. In contrast, cells transformed with EGFP alone presented a diffused cytoplasmic fluorescence.

Cd tolerance

Drop-test experiments showed that the expression of AtHMA4 was able to increase the tolerance up to 150 μ M Cd (Fig. 4A). In contrast, expression of the mutant forms *Athma4as* and *Athma4 Δ His* did not change the wild-type phenotype (Fig. 4A). As previously reported, the *ycf1* strain was extremely sensitive to Cd (Fig. 4A) (Li et al., 1996; Mason and Michaelis, 2002). When the *ycf1* strain was transformed with *AtHMA4*, the tolerance to Cd was restored to the level of the wild-type (Fig. 4A). This result shows that AtHMA4 functionally complements the *ycf1* defective strain. The two truncated forms *Athma4as* and *Athma4 Δ His* were unable to complement the *ycf1* mutant strain (Fig. 4A). To test whether increased tolerance to Cd and *ycf1* phenotypic complementation was due to transport or solely to a chelation phenomenon, a D401A mutant of AtHMA4 (*Athma4D401A*) was generated. Asp-401 belongs to the strictly conserved motif in all P-ATPases, DKTGT, and is predicted to be the invariant residue phosphorylated in the activated enzyme. Thus the transport function must be impaired in the *Athma4D401A* mutant. Indeed, this mutant did not present any increased tolerance to Cd nor complementation of the *ycf1* mutant strain (Fig. 4A). This experiment clearly demonstrates that the detoxification process of

yeast cells by AtHMA4 is grounded to a transport function. Liquid culture experiments carried out in the same range of cadmium concentrations used in drop-tests confirmed the results obtained on plates. Similar experiments were carried out at 70 μ M cadmium but in the presence of 5 mM buthionine sulfoximine (BSO), a known
 5 inhibitor of the γ -glutamylcysteine synthase (Griffith and Meister, 1979). In this case, the *ycf1* strain growth was completely inhibited while wild-type-pYES2 and *ycf1*-*YCF1* strains grew slightly (Fig. 4B). Interestingly, the strain expressing AtHMA4 grew at the same rate in the presence or absence of BSO (Fig. 4B). This result shows that, contrary to YCF1, AtHMA4 does not need a glutathione S-conjugated form of
 10 the metal to detoxify Cd.

AtHMA4 expression in the yeast *S. cerevisiae* induces an increased tolerance to Cd (Fig. 4A) as previously described by Mills et al. (2003). In the presence of 150 μ M Cd, the growth of the wild-type strain was rapidly impaired, whereas *AtHMA4*-transformants were still able to grow. It was no more the case when
 15 Asp-401 was replaced by Ala in AtHMA4. Asp-401 belongs to the phosphorylation consensus domain DKTGT and such mutation impairs for the transport function of P-type ATPases (Moller et al., 1996). As expected, AtHMA4D401A does no longer confer an increased tolerance to Cd nor a phenotypic complementation of the *ycf1* mutant strain. This observation invalidates a chelation-based detoxification process
 20 and demonstrates a strong coupling between metal tolerance conferred by AtHMA4 and hydrolysis. In *S. cerevisiae* yeast cells, the importance of YCF1 in the Cd detoxification process has been clearly demonstrated (Li et al., 1997). This protein, a member of the ATP-binding cassette family, is involved in the transport of glutathione S-conjugated Cd to the vacuole. In accordance with this activity is that the sensitivity
 25 of the *ycf1* mutant strain to Cd is strongly enhanced. Interestingly, the instant experiments demonstrate that AtHMA4 expression was able to neutralise the extrasensitivity to Cd of the defective strain. As expected, in the presence of cadmium and BSO, an inhibitor of γ -glutamylcysteine synthase (Griffith and Meister, 1979), the growth of the wild-type yeast was largely inhibited while the *ycf1* defective strain did not grow
 30 (Fig. 4B). In both strains, AtHMA4 expression restored the growth capability, demonstrating that AtHMA4 was able to preserve the yeast cells from Cd poisoning in a non GS₂-conjugated form.

Pb / Zn / Co tolerance

Similar experiments to those described in the previous section were performed with the 3 other metals (Pb, Zn and Co) that AtHMA4 was predicted to transport. Figure 5 presents the results obtained in the presence of Pb acetate supplied in solid medium. For *AtHMA4*-transformants and vector-only transformants, the same capabilities of resistance were observed, up to a concentration of about 20 mM Pb. As recently published by Song et al. (2003), it was also observed that the *ycf1* mutant strain was highly sensitive to Pb. When AtHMA4 was expressed in the *ycf1* mutant context, it was able to partially restore the growth capability of the mutant strain while the empty vector was not able to (Fig. 5).

A series of drop-test experiments was also carried out in presence of a range of Zn concentrations (Fig. 6). AtHMA4-transformants were slightly more resistant to Zn, up to a concentration of 25 mM, than the wild-type strain. Such increased tolerance was not observed for strains expressing *Athma4as* and *Athma4ΔHis*. Interestingly, AtHMA4 was also able to rescue *zrc1*, a deletion mutant of the zinc vacuolar transporter belonging to the CDF family. As already described, this mutant strain was extremely sensitive to Zn (MacDiarmid et al., 2003). AtHMA4-transformants of this strain, but not *Athma4as* nor *Athma4ΔHis* ones, exhibited a wild-type resistance to Zn.

While AtHMA4 was able to confer a tolerance to Cd, Pb and Zn concentrations, such a phenomenon was not observed in the presence of Co. The *cot1* mutant, defective for the corresponding CDF vacuolar transporter, is known to be very sensitive to Co (Conklin et al., 1992). However, AtHMA4 expression in this mutant strain was unable to restore the Co tolerance.

Other metals were also screened (Ag, Cu, Ni and Fe) but no change in tolerance to these metals was observed when AtHMA4 was expressed, which is in agreement with predictions (Argüello, 2003).

Recently, it has been reported that YCF1 also participates in resistance to Pb toxicity (Song et al., 2003). While AtHMA4 expressed in the *S. cerevisiae* wild-type strain did not enhance Pb resistance, it was able to restore Pb tolerance in the *ycf1* strain (Fig. 5). The instant experiments are the first demonstration of a plant P_{1B}-ATPase conferring Pb detoxification properties. Among P_{1B}-ATPases, only ZntA

from *E. coli* was demonstrated to confer a tolerance to Pb (Rensing et al., 1997 and 1998). Until now, NtCBP4, which belongs to the cyclic nucleotide-gated ion channel (CNGC) family, was the only example of a plant transporter involved in Pb tolerance. The overexpression of this transporter induces Pb accumulation and hypersensitivity
 5 (Arazi and al., 1999). In contrast to this hypersensitivity response, a truncated form, deleted from the C-terminal part, induced an improved tolerance to Pb (Sunkar et al., 2000). The *Arabidopsis* homologue of this tobacco protein, AtCNGC1, exhibited a similar function (Sunkar et al., 2000).

Besides Cd and Pb, AtHMA4 also confers an increased Zn tolerance
 10 (up to 25 mM) and a partial complementation of the defective mutant strain *zrc1* (Fig. 6). This last observation is in contradiction with previous results from Mills et al. (2003) who failed to observe any change in Zn resistance for this yeast strain transformed with *AtHMA4*. On the other hand, these authors observed a functional complementation of *zntA*, an *E. coli* strain defective for the Zn/Cd P-type ATPase
 15 efflux pump. The different experimental procedures and / or the range of Zn concentrations used could explain these discrepancies.

Finally, the ability of AtHMA4 to confer Co tolerance was investigated. AtHMA4 was found unable to induce an increased tolerance nor to restore growth of the yeast defective mutant *cot1* (a Co transporter belonging to the
 20 CDF family; Conklin et al., 1992). Among the CPx-ATPases, CadA from *Bacillus subtilis* confers a moderate tolerance to Co (Gaballa and Helmann, 2003). It is worthy to note that CadA possesses in its N-terminal part the CxxC motif, which could be the binding site of Co, while such a motif is absent in AtHMA4.

Cadmium accumulation (detoxification process in yeast)

25 After a 48 h period in the presence of 40 or 80 μ M Cd, the Cd contents in the wild-type strain and the complemented strains were determined by ICP-AES. The level of Cd accumulated by the yeast was found to be approximately similar for the pYES2-only transformants and the pYES2-*YCF1*-complemented *ycf1* (Fig. 7). When AtHMA4 was expressed in the wild-type strain, the Cd content was
 30 drastically diminished compared to the level found in pYES2-only transformants (a fall of 50 and 62 % of the Cd content for yeast grown in the presence of 40 and 80 μ M Cd, respectively). When AtHMA4 was introduced into the *ycf1* context, the fall in Cd

content of the yeast cells was even more pronounced (80 and 83 % compared to *ycf1*, for yeast grown in the presence of 40 and 80 μ M Cd respectively).

To limit Cd toxicity, AtHMA4 could participate in i) Cd efflux processes at the yeast plasma membrane ii) Cd transport to the vacuole iii) Cd chelation aim to the large polyhistidine motif at the C-terminal part of the protein. The results obtained with either *AtHMA4D401A* and the spliced form of AtHMA4 let us to turn down this last hypothesis. Indeed, a simple chelation process seems unlikely since these forms of the protein exhibit the polyhistidine stretch but failed to modify yeast Cd tolerance and content. The deletion in the alternative spliced form is located in the central part of the protein corresponding to the large cytosolic loop, near the strongly conserved motifs MLTGDN and GDGVNDAP. This last motif is part of the putative hinge domain (Scarborough, 2000; Xu et al., 2002). Such deletion of 28 residues probably induces large perturbations in the conformational changes occurring during the catalytic process. All these results suggest that AtHMA4 is involved in an active process of Cd translocation. A Cd transport to the vacuole, mimetic to YCF1 function, would lead to an equal or even increased Cd content in the yeast cells expressing AtHMA4, relative to the wild-type strain. On the contrary, the determination of metal content indicated a drastic decrease in the Cd content in AtHMA4-transformed yeast cells compared to that in the wild-type or YCF1-transformed strains (Fig. 7). These results strongly argue for a role of AtHMA4 in Cd efflux from the yeast cell.

The decrease in the Cd content of yeast strains expressing AtHMA4 suggests a plasma membrane location of the protein which would allow an efflux of the toxic towards the external medium. However, fluorescence imaging of the AtHMA4::EGFP did not detect high levels of the protein at the plasma membrane nor at the vacuolar membrane (Fig. 3B). The punctuated staining pattern observed appears typical of vesicles from the late Golgi which traffic to the plasma membrane, as previously described (Yuan et al, 1997; Nothwehr et al., 2000; Weissman et al., 2002). Thus, we propose that AtHMA4 participates in Cd loading in late Golgi vesicles driving Cd efflux at the yeast plasma membrane.

Finally, the AtHMA4DHis form of the protein was found non functional in metal tolerance experiments, suggesting that the polyhistidine motif is essential in the binding and / or translocation of metal. In *Haemophilus*, the His-rich

domain in the N-ter part of the Cu,Zn superoxide dismutase has been shown to initially bind the metals to be delivered to the active site (Battistoni et al. (2001). The polyhistidine stretch at the C-terminal part of AtHMA4 could play a similar role of “self chaperone”.

5 **Tissue-specific expression of *AtHMA4* in Arabidopsis**

The level of expression of *AtHMA4* in various organs of Ws ecotype was investigated by RT-PCR with part of the *ACTIN8* gene as a control. A 442 bp fragment in the full version of the cDNA and a 358 bp product in the case of the *Athma4as* transcript were amplified. The *AtHMA4* transcript was detected in all
10 tissues analysed with higher expression levels in roots, stems and flowers (Fig. 8). Expression of *Athma4as* was observed in all organs studied though to a lesser extent compared to *AtHMA4*.

T-DNA insertional mutant study

In the line Rm396, a T-DNA is inserted in the 3rd intron (Fig. 9A).
15 Southern hybridization with a probe corresponding to a fragment of the *GUS* gene showed that this insertion was unique; PCR analysis, using primers designed on *AtHMA4* (9HMA4 : 5'-CCATTAAAAGGCCTAGGATCGACATC-3' (SEQ ID NO:21)) and the T-DNA, demonstrated that the mutant line Rm396 was homozygote for the insertion. The absence of an *AtHMA4* transcript was controlled by RT-PCR, using 3
20 different oligonucleotide pairs positioned upstream, downstream and on both sides of the insertion, respectively. As expected, on wild-type Ws RNA extract, two amplification fragments of 708 bp and 574 bp were observed using 1HMA4 (SEQ ID NO:7)/Rev18HMA4 (SEQ ID NO:14) and 10HMA4 (SEQ ID NO:15)/Rev8HMA4 (SEQ ID NO:16) primer pairs, respectively. These fragments were absent when Rm396 RNA
25 extract were used (Fig. 9B, middle and lower parts). The deletion of 7 of 9 coding exons of the *AtHMA4* gene in the Rm396 line makes it likely that AtHMA4 is non functional in this mutant.

GUS expression under standard conditions

Interestingly, the Rm396 mutant presents a translational fusion of
30 the *GUS* gene with the two first exons of *AtHMA4*. Figure 9B (upper part) shows a RT-PCR using a primer pair located upstream of the insertion. In this case, the reverse transcription reaction was realised with the gene specific primer Rev6HMA4 (SEQ ID

NO:1). An amplification fragment was observed with Ws and Rm396 RNA, confirming the existence of a transcript corresponding to the first two exons of *AtHMA4* and the *GUS* gene. This fusion allows detection of GUS activity under the control of the *AtHMA4* promoter. GUS staining was observed in roots, around the phloem and xylem vessels, whatever the state of development of the plant (from 4 days after germination until 8 weeks-old plant) (Fig. 10a). At the level of the roots, GUS expression was particularly intense in pericycle and cambium cells (Fig. 10b). A GUS staining was also observed in the vascular tissues, under the shoot apical meristem, in the stamens and at the bases of cauline leaves (Fig. 10c-e). These results are in good agreement with those obtained by RT-PCR (Fig. 8).

GUS expression in the presence of metals

When plants were exposed for 24 h to Zn concentrations up to 1 mM, a rise in the expression level of *AtHMA4* was observed in the roots (Fig. 11A). Such an up-regulation of the expression level of *AtHMA4* by Zn has been reported by Mills et al. (2003) using RT-PCR with RNA extracts from the Col-0 ecotype. Depending on the Zn concentration applied, a change in the GUS staining intensity and pattern was observed (Fig. 11B). Strikingly, when plants were grown at a high Zn concentration (1 mM), the GUS staining was strictly limited to the elongation zone of the roots (Fig. 11C). In contrast with Zn, Cd and Pb did not change the expression profile whatever the concentrations used, as probed by RT-PCR and GUS staining (data not shown). These last results disagree with a down-regulation of the expression of *AtHMA4* by Cd described in Col-0 (Mills et al., 2003).

Rm396 phenotypic characterization

Since Rm396 was characterized as a knock-out mutant for *AtHMA4*, it was searched for a phenotype associated with metal uptake or metal toxicity. Young plants of Ws ecotype and line Rm396 were grown *in vitro* in the presence of a range of metal concentrations from starvation to toxicity (Cd, Zn, Co, Pb, Cu, Ni, Fe). No significant differences were observed in germination levels and plant development between the wild-type and the mutant line whatever the metals studied (data not shown). Additionally, the analysis of phenotypic characters linked to Cd toxicity (decrease in fresh weight, leaf chlorosis and Cd accumulation in roots and leaves) gave no significant differences between the wild-type and the Rm396 line.

***AtHMA4* expression in Arabidopsis**

By RT-PCR, it has been shown that *AtHMA4* is expressed in all major tissues in the Ws ecotype (Fig. 8), in accordance with observations in the Col-0 ecotype. The expression pattern of *AtHMA4* was studied in details by GUS staining, using the T-DNA insertional mutant from the INRA Versailles collection. GUS staining was mostly observed in roots in the region of the pericycle and cambium cells (Fig. 10). GUS staining was also associated with vascular tissues located near the shoot apical meristem, the stamens and at the bases of cauline leaves. Such localization strongly suggests that *AtHMA4* could be involved in substrate transfer to or from the vascular tissues.

The presence of an alternative spliced form of the *AtHMA4* transcript, specific to the Ws ecotype, leading to a 84 bp deletion and keeping the reading frame (Fig. 2) was observed. This is the first observation of an alternative splicing for a transcript of a plant transporter. By RT-PCR, this alternative spliced form was found at a lower amount than the full-size one in all tissues studied. Several animal genes have been identified that have tissue or developmental stage-specific alternative splicing (for a review see Green, 1991). In the case of the P_{1B} -ATPases, and notably both human genes *ATP7A* (Menkes protein) and *ATP7B* (Wilson protein), they have been found in different alternative spliced forms which could account for the different subcellular distributions of these two proteins (Yang et al., 1997; Qi and Byers, 1998). In contrast, in plants, alternative splicing has been rarely documented but some results indicate that it has an important role. Rubisco activase represents one of the best-known examples in which alternative splicing allows a fine-tuning of the light regulation of the enzyme (Werneck et al., 1989). A second example is the *FCA* transcript alternative splicing which is involved in flowering regulation (Macknight et al., 1997 and 2002). Despite different treatments and experiments have been conducted with a variety of heavy metals, we failed to observe a regulation of this alternative spliced form *in planta* and a functional role in yeast strains. However, the view cannot be excluded that this truncated protein plays a physiological role apart metal detoxification.

Since *AtHMA4* is a putative Zn/Cd/Pb/Co transporter, the effects of such metals on its expression level and patterning were analysed using RT-PCR

experiments and GUS staining. *AtHMA4* expression was up-regulated in roots by Zn for a concentration range up to 1 mM (Fig. 11), in accordance with RT-PCR experiments in the present study and Mills et al. (2003). At the higher Zn concentration (1 mM), a strong GUS staining was observed but restricted to the root elongation region.

5 Strikingly, RT-PCR and GUS staining experiments did not indicate any regulation of *AtHMA4* expression by Cd or Pb, which is in contrast to previous results from Mills et al. (2003). The known differences in Cd tolerance between Ws and Col-0 ecotypes, and in the expression profiles found in Ws and Col-0, may be involved in these divergent observations. Furthermore, these studies were conducted with different salts of

10 Cd (CdCl_2 in this work vs. CdSO_4 in other work), and the associated anion may create the different results.

A defective mutant of *AtHMA4* does not exhibit any specific phenotype under normal or metal excess conditions and such an absence of phenotype could result from the presence of other transporters, belonging to the same (such as

15 *AtHMA2* and *AtHMA3*) or to other protein families, and supporting redundant functions. This is illustrated in the yeast since some *AtHMA4* properties were only observed in a mutant context.

Overexpression of *AtHMA4*

To test the role of *AtHMA4* in *Arabidopsis*, plants ectopically over-

20 expressing this gene were generated. The *AtHMA4* cDNA was cloned under the strong constitutive CaMV35S promoter and introduced in *Arabidopsis* through *Agrobacterium tumefaciens*-mediated transformation. Two independent lines with elevated *AtHMA4* mRNA levels (Fig. 12A) carrying single T-DNA insertion were selected and self-crossed to obtain stable homozygous lines. The Zn content of shoot

25 from overexpressing plants, hydroponically grown on nutrient solution (3 μM Zn) was measured by ICP-AES. *AtHMA4* leaves from overexpressing plants accumulated 61 % more Zn than the control (Fig. 12B). Both overexpressing lines displayed Cd and Zn increased tolerance as assayed by root growth measurements (Fig. 12C and D). On control solid medium (Zn 3 μM), root lengths of the overexpressing plants were not

30 statistically different from the one of the wild-type. However, on media containing high Zn concentrations (50, 200 μM) or Cd (10, 40 μM), the root lengths from

overexpressing lines were greater by 35, 154, 43 and 123 % compared to wild-type roots, respectively.

Despite it was failed to find a phenotype for the knock-out mutant, overexpression of AtHMA4 *in planta* lead to an increased tolerance to Zn and Cd (Fig.12 D), as already observed in the yeast. This increased tolerance was particularly obvious at the level of the root development during a Zn or Cd treatment (as an example, the root was two times longer for the overexpressing lines in comparison to the wild-type plants in presence of Cd 40 μ M). When grown in control conditions, the overexpressing lines did not present longer roots, but the Zn content was 61 % higher than the one of the wild-type.

Altogether these observations strongly suggest that AtHMA4 participates in the loading of heavy metal in the xylem, allowing their translocation to the shoot.

The same may be observed for AtHMA1-3.

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CLAIMS

1°) Genetically modified plants, characterized in that they include more than one copy of at least a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass and that they overexpress said P_{1B}-type ATPase.

5 2°) Genetically modified plants according to claim 1, characterized in that said P_{1B}-type ATPase is selected from the group consisting of heavy metal ATPase HMA1, HMA2, HMA3 and HMA4 of *Arabidopsis thaliana*.

3°) Genetically modified plants according to claim 1 or claim 2, characterized in that said genetically modified plants include more than one copy of at
10 least two different sequences encoding two different P_{1B}-type ATPases of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass.

4°) Genetically modified plants according to claim 1 or to claim 2, characterized in that they include more than one copy of a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass and at least another sequence
15 selected among sequences encoding (1) an enzyme involved in metal chelation (phytochelatin synthase, glutathion synthetase or gamma-glutamylcystein synthase) or (2) another metal transporter such as YCF1 or other ABC transporters.

5°) Recombinant vector able to transform plants, characterized in that said vector includes more than one copy of at least a sequence encoding a P_{1B}-
20 type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass.

6°) Recombinant vector according to claim 5, characterized in that said coding sequences are operably linked to and under the regulatory control of a plant-expressible transcription and translation regulatory sequence, such as a plant specific promoter.

25 7°) Genetically modified plants, characterized in that they are transformed with a recombinant vector according to claims 5 or 6.

8°) Plant cells characterized in that they are transformed with a recombinant vector according to claims 5 or 6.

9°) Method of producing genetically modified plants according to
30 claims 1 to 4 or 7 which overexpress at least a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass, said method comprising:

- preparing a recombinant vector according to claims 5 or 6, comprising more than one copy of at least a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass, operably linked to and under the regulatory control of a plante-expressible transcription and translation regulatory sequence and

5 - introducing said recominant vector into a plant cell or plant tissue to produce a genetically modified plant cell or a genetically modified plant tissue.

10°) Method of phytoremediation of heavy metals from soil, characterized in that it includes:

10 - a step of planting genetically modified plants according to any one of claims 1 to 4 or 7, in an area containing soil contaminated with at least one heavy metal and

 - collecting and removing plant tissues from said genetically modified plants at appropriate time intervals.

15 11°) Method of phytoremediation according to claim 10, characterized in that it involves the extraction of one of the following heavy metals: Zn, Co, Cd or Pb, from soil.

 12°) Method of phytoremediation according to claim 10 or claim 11, characterized in that the entire plant might be removed after it is allowed to grow on metal-containing soil incorporating those metals into its tissues.

20 13°) Method of phytoremediation according to claim 10 or claim 11, characterized in that at appropriate time intervals, the metal containing tissues and more preferably leaves and possibly branches are removed from the plant, allowing the remaining plant tissues to survive.

 14°) Method of phytoremediation according to claim 13, 25 characterized in that the collected plant tissues are removed from the growing area and properly disposed, so that the metal containing tissues are not allowed to reassimilate in the soil.

 15°) Method of phytoremediation according to claim 13, 30 characterized in that said heavy metals may be extracted, in the Mⁿ⁺ state, from said plant tissues.

16°) Method of phytoremediation according to claim 13, characterized in that said heavy metals are extracted from ashes obtained after having burnt the collected metal containing tissues, said metal being in the M^0 state.

17°) Genetically modified plants, as defined in any one of claims 1
5 to 4 and 7, characterized in that said plants are selected in the group consisting of *Brassica juncea*, *Poplar*, *Nicotiana tabacum*.

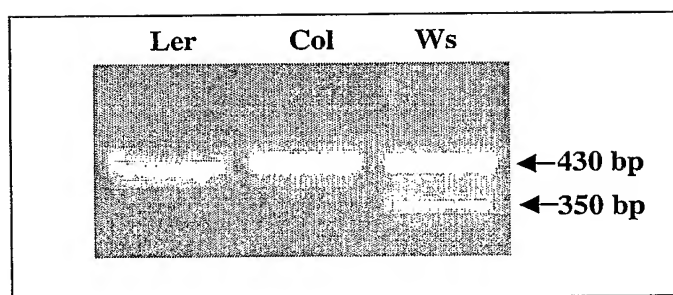
ABSTRACT

Genetically modified plants able to accumulate heavy metals in shoots and methods of removing and possibly recovering said heavy metals, using said genetically modified plants.

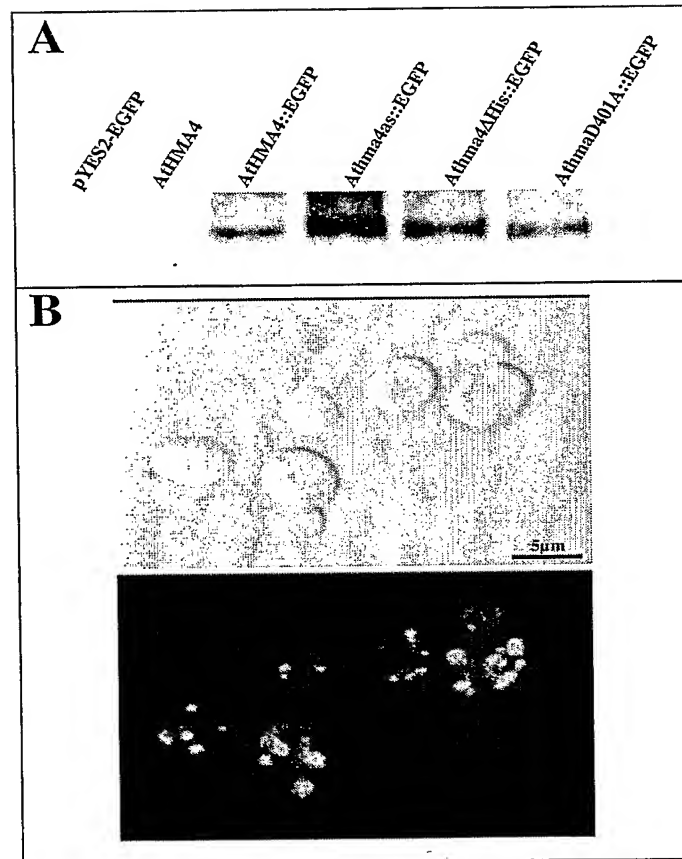
- 5 Said genetically modified plants include more than one copy of at least a sequence encoding a P_{1B}-type ATPase of the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subclass and that they overexpress said P_{1B}-type ATPase.

Figure 1

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**Figure 2**

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**Figure 3**

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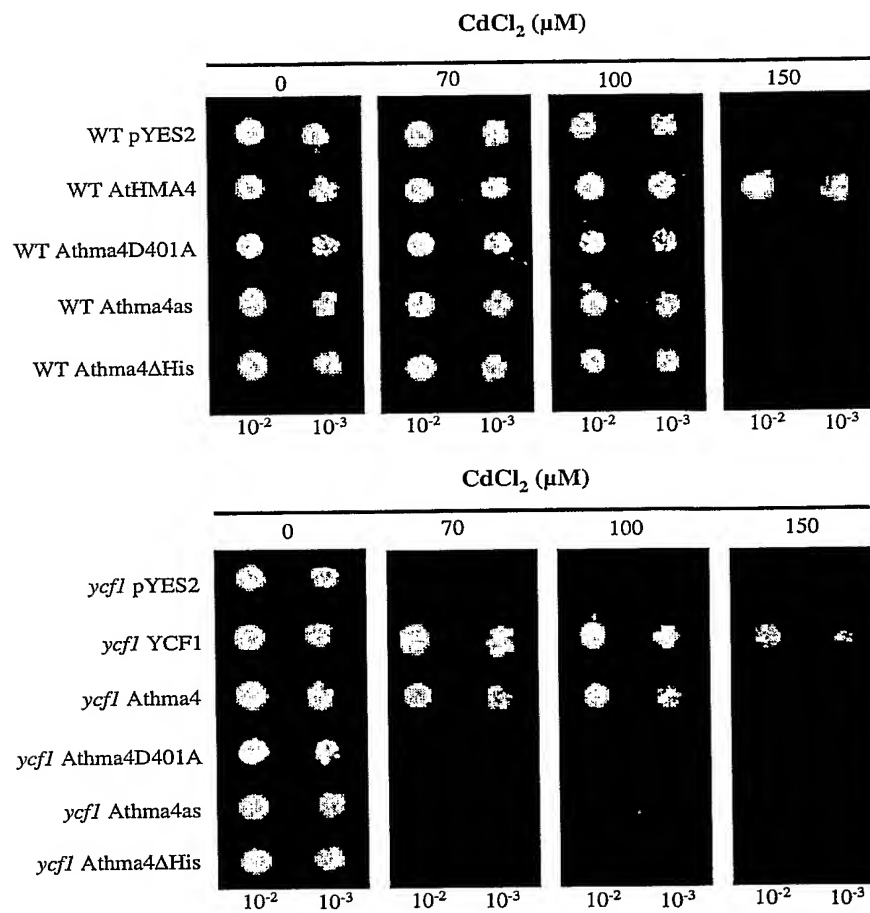


Figure 4A

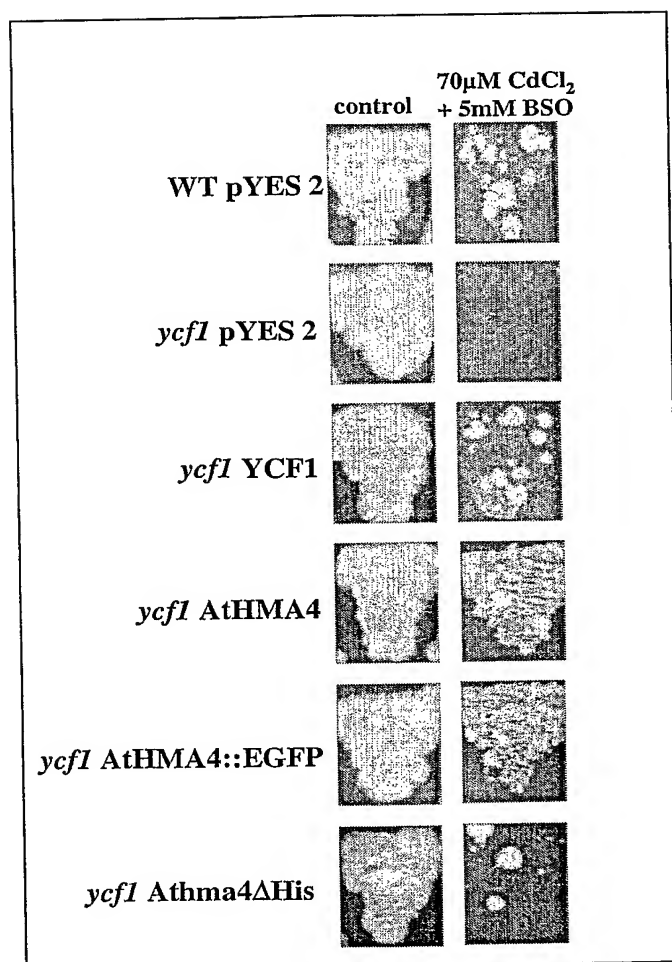


Figure 4B

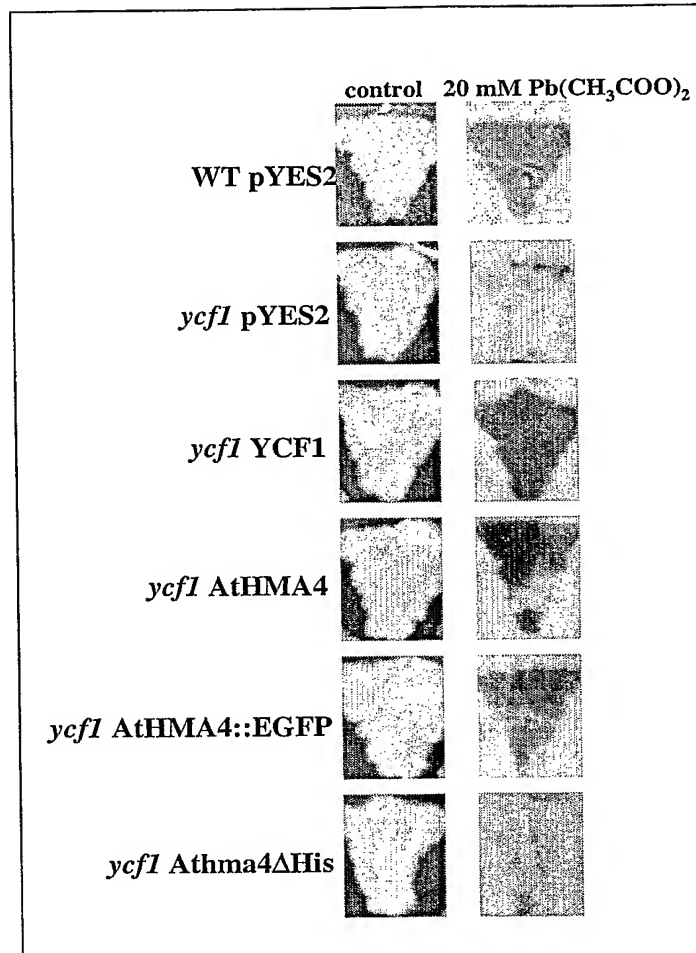


Figure 5

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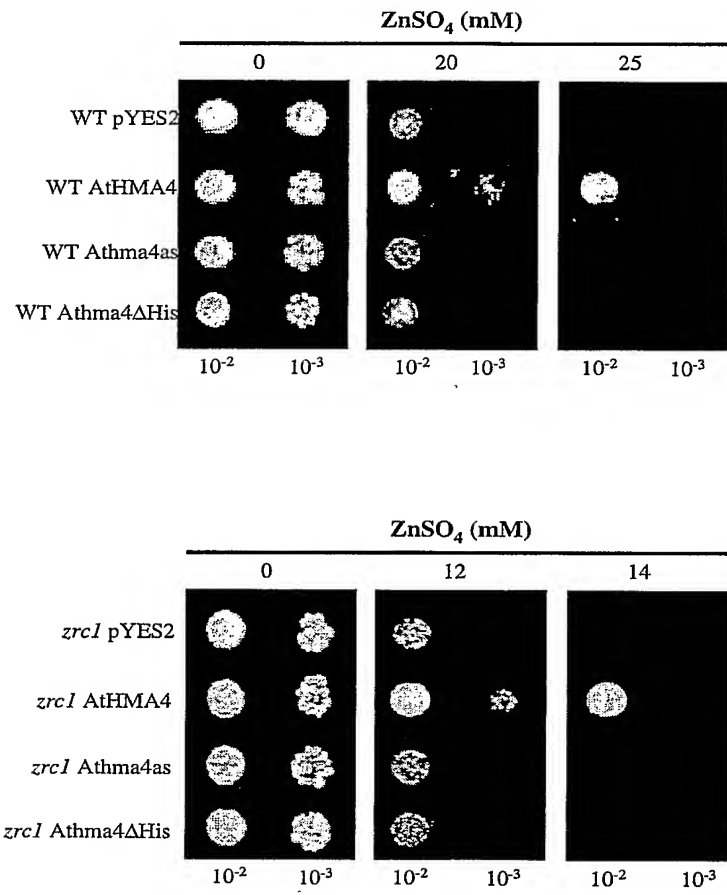


Figure 6

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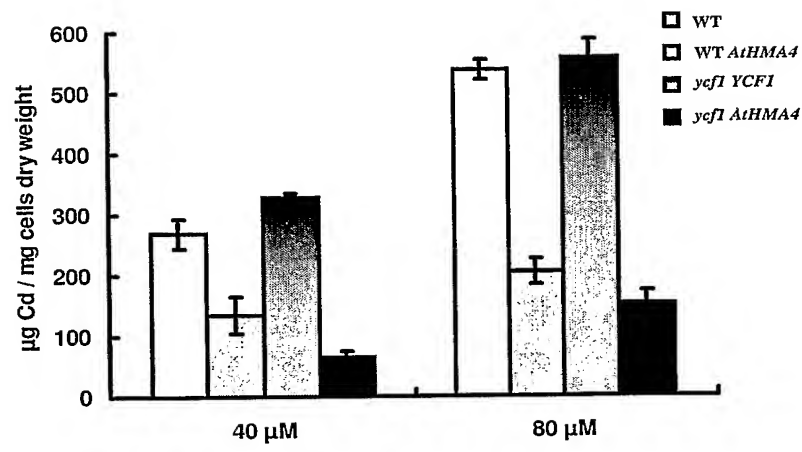


Figure 7

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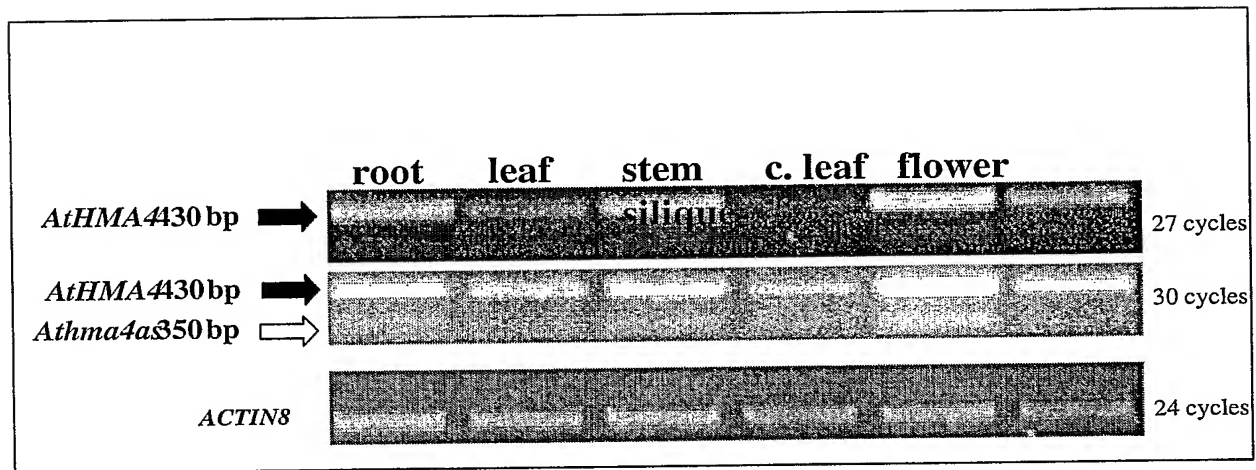


Figure 8

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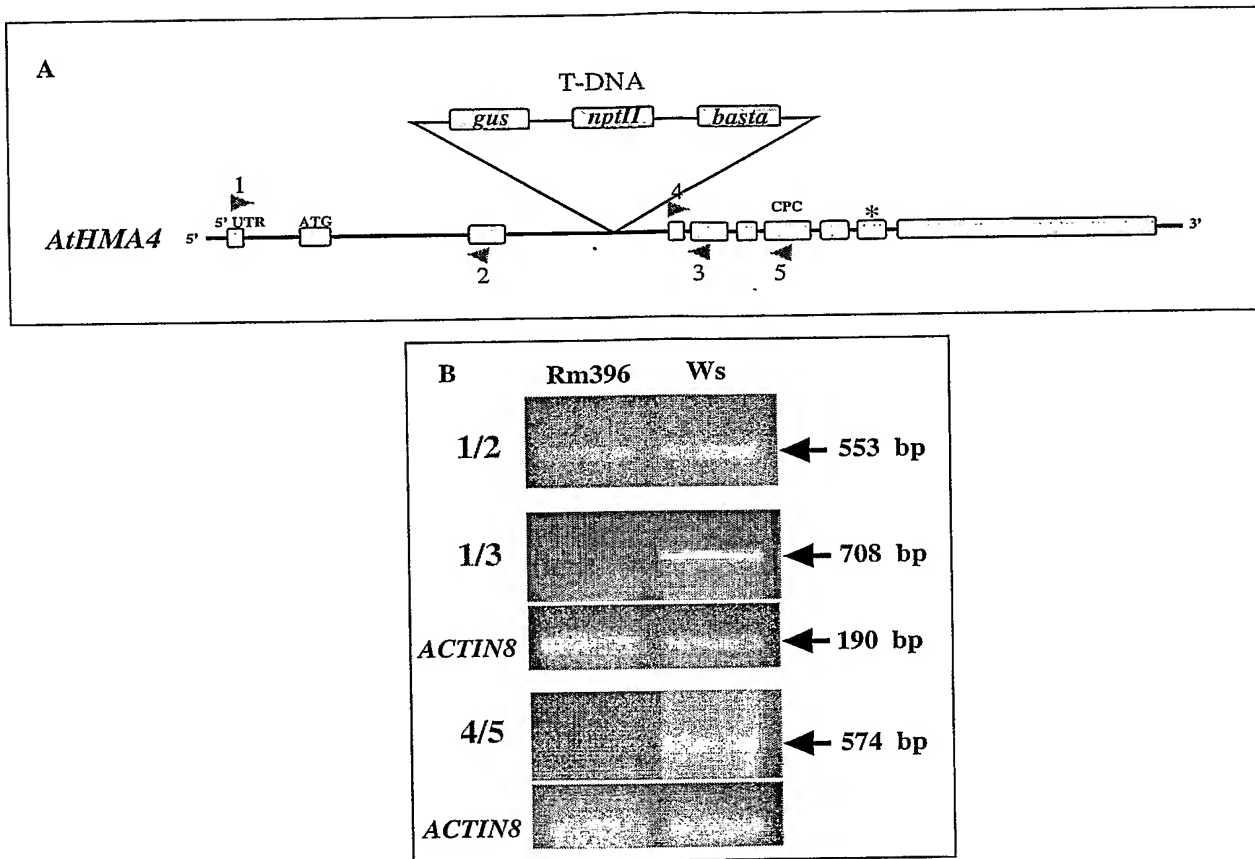


Figure 9

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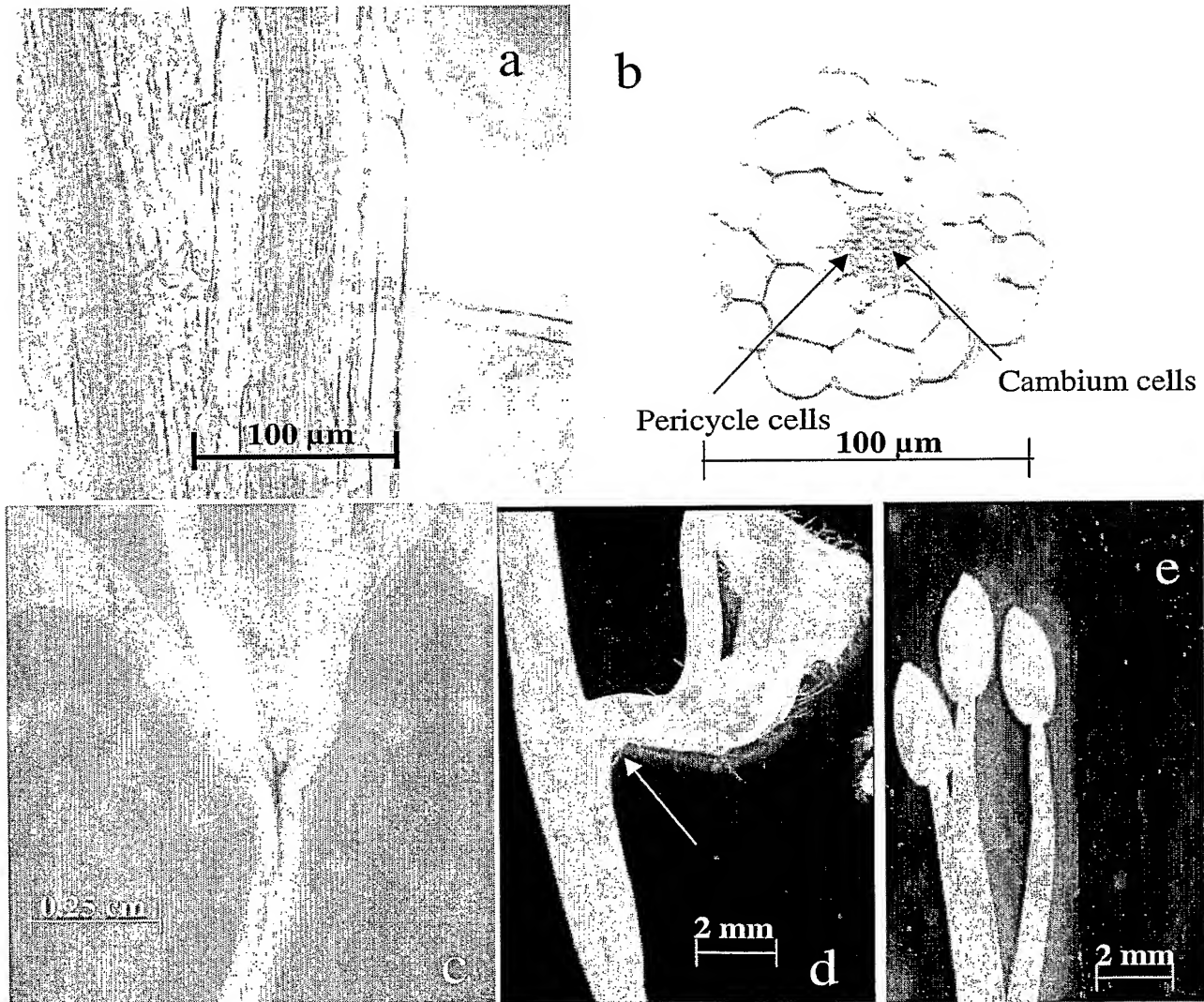


Figure 10

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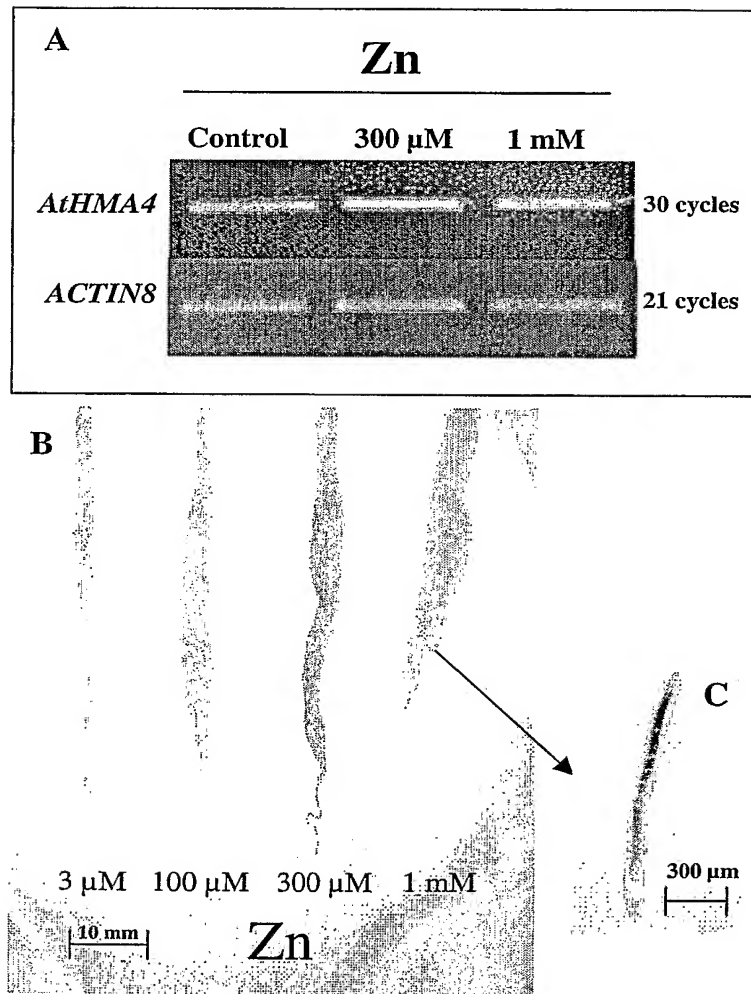


Figure 11

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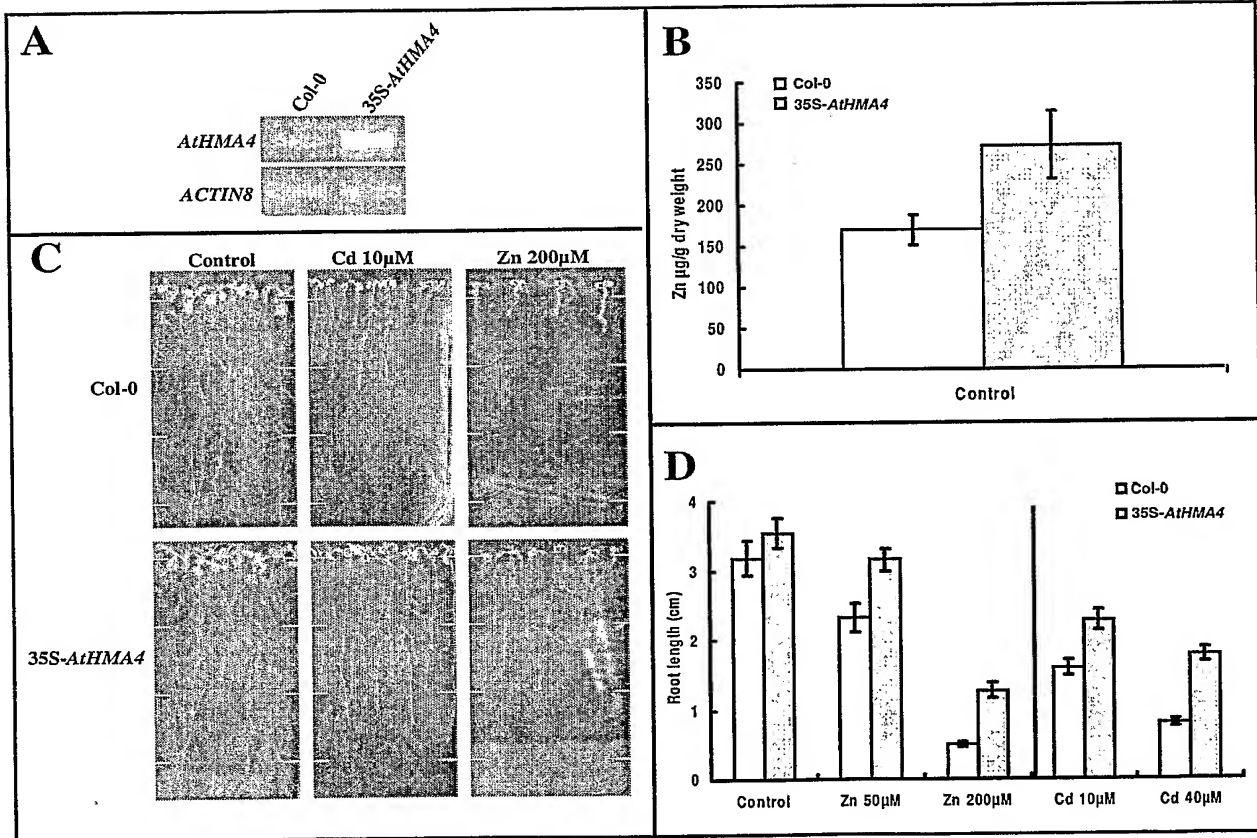


Figure 12

0263-113-SEQ.ST25
SEQUENCE LISTING

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VERRET, Frédéric
GRAVOT, Antoine
AUROY, Pascaline
VAVASSEUR, Alain
RICHAUD, Pierre

<120> Genetically modified plants and their applications in phytoremediation

<130> BLOcp263/113

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0263-113-SEQ.ST25

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